

EFFECT OF RIBAVIRIN ON VIRAL HEPATITIS IN LABORATORY ANIMALS

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Human viral hepatitis has continued to be of major concern to public health authorities because of its relative high rate of incidence throughout the world.¹ The disease is generally regarded as being at least two entities, Type A (infectious) hepatitis and Type B (serum) hepatitis, caused by separate, as yet not fully characterized, viruses. It is unfortunate that despite the severe public health problem of these diseases, experimental systems that could be used to evaluate potential chemotherapeutic agents for treatment of hepatitis are virtually nonexistent.

The broad-spectrum antiviral activity²⁻⁶ of the triazole nucleoside 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) suggested possible utility against viruses causing hepatitis. This potential efficacy was further enhanced by the finding that the orally administered drug can be recovered in significant quantities, > 40% and > 5%, in liver and spleen, respectively, of mice and rats within 30 to 60 minutes of treatment.⁷ Experiments were therefore undertaken to attempt to demonstrate ribavirin's efficacy against experimental infections induced by two widely differing viruses that cause hepatitis in laboratory animals. The results of these experiments are described in this report.

MATERIALS AND METHODS

Viruses

Viruses used in these studies were an RNA coronavirus, the Friend-Braunsteiner strain of murine hepatitis⁸ (MHV) obtained from the American Type Culture Collection (Rockville, Md.), and a DNA herpesvirus, equine abortion virus^{9,10} (EAV) provided by Dr. P. E. Came, Schering Corp. (Bloomfield, N.J.). Both viruses were prepared as liver homogenates after two intraperitoneal (i.p.) passages in the pertinent host animals.

Drug

Ribavirin was synthesized in these laboratories,³ and used in a sterile physiological saline solution.

Animals

Male Swiss Webster mice (Hilltop Lab Animals, Inc., Chatsworth, Calif.) weighing 15-18 g, and female Syrian golden hamsters (Lakeview Hamster Colony, New-

field, N.J.) weighing 40–50 g were used in the studies with MHV and EAV, respectively.

EAV Studies

Individually caged hamsters were inoculated i.p. with approximately 10 times a 50% lethal dose (LD_{50}) of EAV. Ribavirin or saline only was administered i.p. 1 hr before virus inoculation and continued twice daily for 4 days. The animals were observed daily for death. Two dosages of the drug, 200 and 100 mg/kg/day, were used. This experiment was later repeated using 400 and 300 mg/kg/day. In another experiment, a single i.p. injection of 500 mg/kg of ribavirin was given within 10 min after virus inoculation. Ten infected hamsters were used in each group receiving ribavirin, and 20 infected animals received saline as virus controls. Five uninfected hamsters were similarly treated to serve as toxicity controls.

MHV Studies

Mice were inoculated i.p. with approximately 10 LD_{50} of MHV for this series of experiments. In the first study, ribavirin in doses of 75, 37.5, and 18.8 mg/kg/day was administered i.p. to the animals twice daily for 9 days, beginning 2 hr before virus inoculation. The experiment was later repeated using drug dosages of 18.8, 9.4, and 4.7 mg/kg/day. As with the EAV studies, ten infected animals were used in each drug treatment group, with 20 used as virus controls and five for each toxicity control group.

Friend *et al.*¹¹ have reported that, in MHV-infected mice, virus replication resulted in liver necrosis and increases in serum glutamic oxaloacetic transaminase (SGOT). An experiment was therefore run to determine the effect of ribavirin treatment on this parameter, as well as on serum bilirubin, on the concentration of virus recoverable from the liver, and on the observable necrosis caused by the infection in the liver. For this experiment, large groups of mice were treated i.p. with saline or ribavirin (20 mg/kg/day) twice daily for 9 days, beginning 2 hr before virus inoculation. On days 1, 3, and 5, five mice per group were removed for collection of blood and livers. SGOT activity was determined colorimetrically using the kit commercially available from Sigma Chemical Co. (St. Louis, Mo.).¹² Serum bilirubin (direct) was likewise measured using the modified Nosslin technique¹³ employing American Monitor Corp. reagents (Indianapolis, Ind.). Virus levels in the liver were determined using death endpoints in hamsters injected i.p. with varying dilutions of the liver homogenates.

RESULTS

EAV Studies

Hamsters infected with EAV and treated with saline began dying on day 3, and by day 7 all had died with a mean survival time of 4.2 days (FIGURE 1). Ribavirin at a dosage of 200 mg/kg/day caused an increase in mean survival time of 2.2 days, which was statistically significant ($p < 0.05$, t-test). Of these ribavirin-treated hamsters, 20% survived the infection. No toxicity was seen at this dose. The lower ribavirin dosage of 100 mg/kg/day was not effective. When the experiment was repeated using

higher ribavirin levels, the 400 mg/kg/day dosage was lethally toxic to the control animals, and the 300 mg/kg/day dosage was approximately 25% lethally toxic. Each dose caused significant ($p < 0.02$) increases in mean survival time in the infected animals. When ribavirin was administered in a single i.p. injection at the time of virus inoculation, a 1.1-day increase in mean survival time was seen, but such an increase was insignificant.

MHV Studies

Ribavirin at the three higher levels was effective in preventing death in the mice (TABLE 1). When the experiment was repeated using altered doses, significant anti-

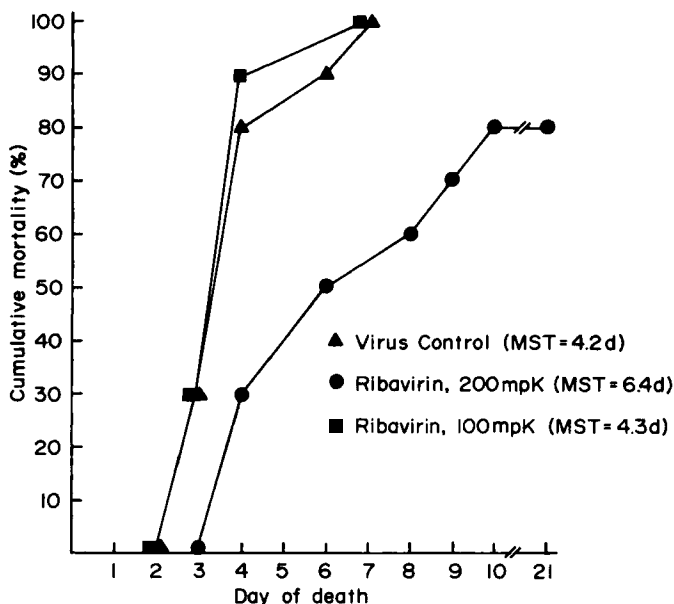


FIGURE 1. Effect of ribavirin treatment on equine abortion virus induced hepatitis in hamsters.

viral effects were again seen, although at the two lowest doses the effect was in the form of significant mean survival time increase only (TABLE 1).

Virus control SGOT levels rose to a high level by day 3 and remained elevated through day 5 (FIGURE 2). By day 7, too many of the animals had died to continue taking samples. Ribavirin treatment delayed the increase of this enzyme, although by day 5, a moderate increase had occurred. Later studies indicated this level soon declined to normal values. Toxicity control animals exhibited no significant alteration in SGOT. Serum bilirubin levels were likewise elevated in the virus control animals through the 5th and last day of the study (FIGURE 3). Ribavirin treatment in both infected and uninfected mice caused an initial moderate rise in bilirubin which then declined by day 3, although in infected, treated mice a rise was seen again on day 5.

Concentrations of MHV in the liver of virus control mice were at relatively high

TABLE I
EFFECT OF RIBAVIRIN TREATMENT* ON MURINE HEPATITIS VIRUS INFECTIONS

Drug Dosage (mg/kg/day)	Tox. Control Surv./Total	Infected, Treated Surv./Total	Surv. Incr. p†	Infected, Treated Mean Surv.‡ Time (Days)	Surv. Time Increase p§
<i>Expt 1</i>					
75	5/5	5/10	0.008	7.2	< 0.001
37.5	5/5	6/10	0.002	6.8	< 0.001
18.8	5/5	7/10	< 0.001	5.7	< 0.05
0		1/20		4.4	
<i>Expt 2</i>					
18.8	5/5	10/10	< 0.001	> 21.0	< 0.001
9.4	5/5	4/10	0.17	6.8	< 0.001
4.7	5/5	3/10	0.28	6.4	< 0.01
0		4/20		4.8	

**b. i. d.* for 9 days, beginning 2 hr before virus inoculation.

†Fisher's exact test.

‡Animals dying on or before day 21.

§Student's t-test.

levels by day 1, increasing to a peak concentration on day 3, but dropping by day 5 (FIGURE 4). This latter decline may be a result of a selecting process, since the majority of the animals had died by day 5, and those remaining alive may have had a lesser initial infection. The maximum virus level attained preceded the mean day of death by about 1.5 days. Ribavirin treatment markedly slowed the production of recoverable virus, although a relatively high titer was seen on day 5.

By days 3 and 5, marked damage was plainly visible in the virus control livers; this

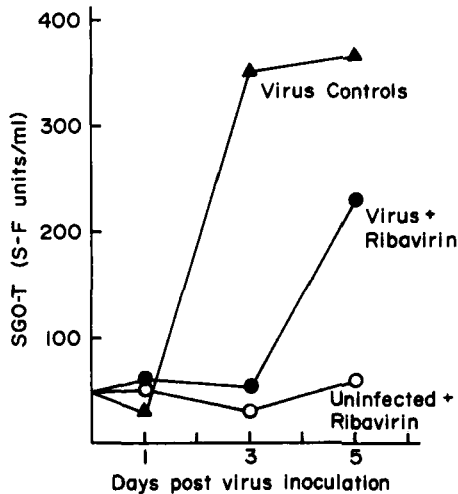


FIGURE 2. Effect of ribavirin treatment on serum glutamic oxaloacetic transaminase levels in mice infected with murine hepatitis virus.

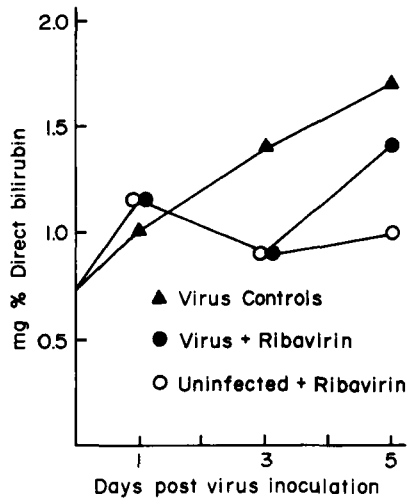


FIGURE 3. Effect of ribavirin treatment on serum bilirubin (direct) levels in mice infected with murine hepatitis virus.

infection was notably less apparent in the ribavirin-treated animals. Scores applied to the degree of liver damage in these mice are summarized in TABLE 2. Liver samples from infected, untreated mice examined histopathologically were found to have moderate hepatitis evidenced by cytoplasmic vacuolation in the form of cloudy swelling and lipidosis. Focal necrosis and hemorrhage were also in evidence.

DISCUSSION

These studies offer considerable evidence that parenterally administered ribavirin has a marked effect on hepatitis in mice induced by MHV, an RNA virus, but that

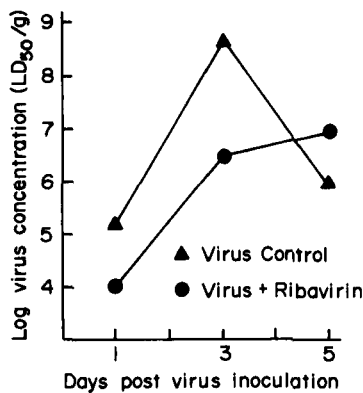


FIGURE 4. Effect of ribavirin treatment on the concentration of hepatitis virus in livers of mice infected with murine hepatitis virus.

the drug, at least by the treatment regimens used in these experiments, has only a marginal influence on hepatitis in hamsters induced by EAV, a DNA virus.

Preliminary results suggest that ketoaldehydes derived from steroid compounds¹⁴ and certain substituted morpholinium quaternary salts¹⁵ may have a moderate efficacy against MHV infections; other drugs showing action against MHV infection include the antihistamine drug Benadryl and the antibiotic Streptothricin. In the study with the antihistamine, the effect was to prevent necrosis of liver cells, rather than to inhibit viral replication. Streptothricin was thought more to exert its effect as a direct action on the virus than to be a therapeutic effect.¹⁷ The action of ribavirin indicates a probable therapeutic and antiviral effect, although additional studies using later initiation of treatment are needed.

Drugs reported to be most effective against EAV infections in hamsters include 9- β -D-arabinofuranosyladenine¹⁸ and 9- β -D-arabinofuranosylhypoxanthine monophosphate.¹⁹

Both MHV and EAV infections were rapidly fulminating to a lethal endpoint; such an infection does not mimic the more slowly progressing, less fatal human hepatitis infections, so it is difficult to ascertain the predictability of either system to the human situation.

TABLE 2
EFFECT OF RIBAVIRIN TREATMENT ON MURINE HEPATITIS VIRUS-INDUCED LIVER DAMAGE IN INFECTED MICE

Treatment	Mean Liver Damage Score*		
	Day		
	1	3	5
Ribavirin (20 mg/kg/day)	0.5	1	1.2
Saline	2.0	3.1	3.7

*Scores of 0 to 4 assigned by visual examination of degree of damage, with 0 = normal liver and 4 = total involvement of liver.

Biochemical studies have indicated that ribavirin may exert its antiviral effect as the 5'-monophosphate by inhibiting enzymes involved in guanosine monophosphate synthesis.²⁰ The drug is readily converted to the 5'-phosphate in the liver and spleen, presumably by adenosine kinase. Such an active involvement of the liver and spleen in the drug's metabolism suggests its possible utility for therapy of hepatitis, since these organs are of primary importance in the early replication of murine hepatitis viruses.¹¹ Oxford²¹ has reported evidence for ribavirin's inhibition of structural and nonstructural polypeptides and antigens of influenza virus, which may be an alternative mechanism of action for the drug against MHV as well.

At present, ribavirin is undergoing clinical trial against acute Types A and B hepatitis and is also being used in an attempt to eliminate Australian antigen positives from chronic human carriers. Early reports suggest definite efficacy against the Type A infections,²² and possibly success in the antigen studies.^{23,24}

REFERENCES

- DIENHART, F. & A. W. HOLMES. 1965. Epidemiology and etiology of viral hepatitis. *In Progress in Liver Diseases*, H. Popper & F. Schaffner, Eds. Vol. 2: 373. Grune, New York, N.Y.

2. SIDWELL, R. W., J. H. HUFFMAN, C. P. KHARE, L. B. ALLEN, J. T. WITKOWSKI & R. K. ROBINS. 1972. Broad-spectrum antiviral activity of Virazole: 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide. *Science* **177**: 705-706.
3. WITKOWSKI, J. T., R. K. ROBINS, R. W. SIDWELL & L. N. SIMON. 1972. Design, synthesis and broad-spectrum antiviral activity of 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide and related nucleosides. *J. Med. Chem.* **15**: 1150-1154.
4. HUFFMAN, J. H., R. W. SIDWELL, G. P. KHARE, J. T. WITKOWSKI, L. B. ALLEN & R. K. ROBINS. 1973. *In vitro* effect of 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) on deoxyribonucleic acid and ribonucleic acid viruses. *Antimicrob. Ag. Chemother.* **3**: 235-241.
5. SIMON, L. N., R. W. SIDWELL, G. P. KHARE, D. G. STREETER, J. P. MILLER, J. T. WITKOWSKI, J. H. HUFFMAN & R. K. ROBINS. 1973. Molecular basis of antiviral chemotherapy: Virazole, a new broad-spectrum antiviral agent. *In Virus Research, 2nd ICN-UCLA Symposium on Molecular Biology*, C. F. Fox and W. S. Robinson, Eds.: 415-436. Academic Press, New York, N.Y.
6. SIDWELL, R. W., J. T. WITKOWSKI & R. K. ROBINS. 1974. Antiviral activity of Virazole: Review and structure-activity relationships. 1974. *Prog. Chemother., Proc. 8th Int. Congr. Chemother.*, Athens, Greece. **2**: 889-903.
7. MILLER, J. P., D. G. STREETER, R. K. ROBINS & L. N. SIMON. 1976. The relationship between the metabolism of ribavirin and its proposed mechanism of action. *Ann. N.Y. Acad. Sci.* This volume.
8. BRAUNSTEINER, H. & C. FRIEND. 1954. Viral hepatitis associated with transplantable mouse leukemia. I. Acute hepatic manifestations following treatment with urethane or methylformamide. *J. Exp. Med.* **100**: 665-677.
9. DOLL, E. R., J. T. BRYANS, W. H. MCCOLLUM & E. W. CROWE. 1956. Propagation of equine abortion virus in Syrian hamsters. *Cornell Vet.* **46**: 68-82.
10. RANDALL, C. C. & E. C. BRACKEN. 1957. Studies on hepatitis in hamsters infected with equine abortion virus. I. Sequential development of inclusion and growth cycle. *Amer. J. Pathol.* **33**: 709-727.
11. FRIEND, C., F. WROBLEWSKI & J. S. LA DUE. 1955. Glutamic-oxalacetic transaminase activity of serum in mice with viral hepatitis. *J. Exp. Med.* **102**: 699-704.
12. Sigma Technical Bulletin No. 505. Sigma Chemical Co., St. Louis, Mo.
13. NOSSLIN, B. 1960. The direct diazo reaction of bile pigments in serum. *Experimental and clinical studies. Scand. J. Clin. and Lab. Invest.* **12** (Suppl. 49): 1-176.
14. CAVALLINI, G., E. MASSARANI & D. NARDI. 1964. Antiviral compounds. IX. Steroid derivatives. *J. Med. Chem.* **7**: 673-675.
15. ANDERSON, E. L., J. E. CASEY, JR., E. E. FORCE, E. M. JENSEN, R. S. MATZ & D. E. RIVARD. 1966. Antiviral agents. II. Substituted morpholinium quaternary salts. *J. Med. Chem.* **9**: 211-213.
16. JUDAH, J. D., G. BJOTVEDT & T. VAINIO. 1960. Protection against liver injury due to murine hepatitis virus. *Nature* **187**: 507-508.
17. STONE, R. L., D. C. DE LONG, R. N. HULL & I. S. JOHNSON. 1965. *In vivo* and *in vitro* inhibition of hepatitis viruses by streptothricin. *Ann. N.Y. Acad. Sci.* **130**: 355-364.
18. LIEBERMAN, M., A. PASCALE, T. W. SCHAFER & P. E. CAME. 1972. Effect of antiviral agents in equine abortion virus-infected hamsters. *Antimicrob. Ag. Chemother.* **1**: 143-147.
19. ALLEN, L. B., J. H. HUFFMAN, G. R. REVANKAR, R. L. TOLMAN, L. N. SIMON, R. K. ROBINS & R. W. SIDWELL. 1975. Efficacy of 9- β -D-ribofuranosylhypoxanthine-5'-monophosphate in therapy of equine abortion virus-induced hepatitis in hamsters. *Antimicrob. Ag. Chemother.* **8**: 474-478.
20. STREETER, D. G., J. T. WITKOWSKI, G. P. KHARE, R. W. SIDWELL, R. J. BAUER, R. K. ROBINS & L. N. SIMON. 1973. Mechanism of action of 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a new broad-spectrum antiviral agent. *Proc. Nat. Acad. Sci. U.S.A.* **70**: 1174-1178.
21. OXFORD, J. S. 1975. Inhibition of the replication of influenza A and B viruses by a nucleoside analogue (ribavirin). *J. Gen. Virol.* **28**: 409-414.
22. ZUNIGA, C. B. A. ALMEIDA, A. C. L. IERVOLINO, I. O. CASTRO, & P. A. A. GALVAO. 1974.

- Acao do l-beta-D-ribofuranosyl-1,2,4-triazol-3-carboxamida no tratamento das hepatites virais agudas. *Rev. Assoc. Med. Brasil* **20**: 386-390.
23. VILELA, M. P., W. A. SIQUEIRA, S. H. S. DOMINGUES, L. D. RODRIGUES & F. S. RODRIGUES. 1974. Comportamento do antigeno Australia nos casos de hepatite por virus tratados com o l-beta-D-ribofuranosyl-1,2,4-triazol-3-carboxamide. *Rev. Bras. Clin. Terap.* **3**: 27-28.